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Zmo0994, a novel LEA-like protein from *Zymomonas mobilis*, increases multi-abiotic stress tolerance in *Escherichia coli*

Jungwoo Yang¹, Ha Eun Kim¹, Young Hoon Jung², Jungyeon Kim¹, Do Hyoun Kim¹, Adrian R. Walmsley³ and Kyoung Heon Kim^{1*} 

Abstract

Background: Pretreatment processes and subsequent enzymatic hydrolysis are prerequisites to utilize lignocellulosic sugar for fermentation. However, the resulting hydrolysate frequently hinders fermentation processes due to the presence of inhibitors and toxic products (e.g., ethanol). Thus, it is crucial to develop robust microbes conferring multi-stress tolerance.

Results: Zmo0994, a functionally uncharacterized protein from *Zymomonas mobilis*, was identified and characterized for the first time. A major effect of Zmo0994 was a significant enhancement in the tolerance to abiotic stresses such as ethanol, furfural, 5'-hydroxymethylfurfural and high temperature, when expressed in *Escherichia coli*. Through transcriptome analysis and in vivo experiments, the cellular mechanism of this protein was revealed as due to its ability to trigger genes, involved in aerobic respiration for ATP synthesis.

Conclusions: These findings have significant implications that might lead to the development of robust microbes for the highly efficient industrial fermentation processes.

Keywords: Abiotic stress, Inhibitors, Multi-stress tolerance, Zmo0994, *Zymomonas mobilis*

Background

Recent climate changes due to the long-term use of fossil fuels have necessitated the development of renewable non-fossil fuels, such as bioethanol, to meet increasing global energy needs [1]. Bioethanol is a product of microbial fermentation of sugars from starch and, more recently, lignocellulosic biomass [2]. Lignocellulosic biomass is generally referred to as non-food biomass and is obtained from other organic sources such as wood, grass, and various wastes, which are recalcitrant to natural degradation [2]. Thus, pretreatment and subsequent enzymatic hydrolysis are prerequisites to

utilizing the lignocellulosic sugar for fermentation [3]. This pretreatment, using such as dilute acid at an elevated temperature, is effective for the hydrolysis of pentose polymers in hemicellulose, increasing the access of cellulases to cellulose fibers [4]. However, the resulting hydrolysate by this diluted acid pretreatment, which is most widely employed on an industrial scale, frequently hinders fermentation processes due to the presence of inhibitors, such as furfural and 5'-hydroxymethylfurfural (HMF) [5]. In addition to the presence of such inhibitors, hydrolysate fermentation on an industrial scale generally occurs under environmental stresses, such as temperature fluctuation, hyper-osmosis, and accumulation of toxic end products that inhibit fermentation, thereby resulting in reduced yield [6]. Although several processes are employed to alleviate these stresses and improve fermentation efficiency (e.g., detoxification

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using activated carbon for HMF) [5], the development of robust microbes with multi-stress tolerance is considered the key to enhancing the efficiency of industrial fermentation processes [7].

To date, various strategies have been applied for the improvement of microbial stress tolerance: (1) single or multi-gene manipulation, using such as multi-drug efflux pumps (e.g., *acrAB-tolC*) [8] and chaperones (e.g., *groESR* and *dnaKJE*) [9]; (2) phenotype(s) screening from random mutagenesis libraries constructed by molecular engineering (e.g., gTME) [10]; (3) adaptive evolution under selective pressure [11]; (4) direct evolution engineering using genome shuffling [12]; and (5) omics-analysis for systems biology, providing an insight into the tolerance mechanism and new targets for further manipulations [13]. Despite the numerous efforts, tolerance engineering remains at the laboratory stage [14]. Furthermore, tolerance engineering of microbes was still focused on genetic manipulation of the target genes from the eubacterial or bacterial kingdoms which are of known or expected function. Thus, it should be worthwhile (1) to explore functionally unknown genes in microorganisms and (2) to examine genes in higher organisms, which maintain life in harsh environments, to construct robust microbes for industrial application [15, 16]. For example, the complete genome sequence of *Zymomonas mobilis* indicates that 32.6% of the 1998 protein-coding genes are still functionally unknown or have no similarity with functionally identified genes [17]. Another example is a functionally unknown protein from plants. Recently, the late embryogenesis abundant (LEA) proteins are revealed to have protective roles against drought, high salinity, and extremely high temperature [18, 19]. Recently, heterogeneous expression of an LEA protein from *Dendrobium officinale* in *Escherichia coli* exhibited increased tolerance against high salinity and heat [20].

In this study, we isolated Zmo0994 from *Z. mobilis*, an uncharacterized protein that exhibits increased abundance in the culture supernatant under stress conditions. To utilize Zmo0994 as a tolerance-engineering tool, tolerance tests were performed against a variety of stresses by expressing the *zmo0994* in *E. coli*. Then, the cellular mechanism of Zmo0994 was investigated through transcriptome analysis and in vivo experiments. Thus, this study has the potential to contribute significantly to the development of tolerant microbes that can improve the efficiency of industrial fermentation processes.

Results

Identification of Zmo0994 overproduced under stresses in *Z. mobilis*

As part of a project originally aimed at identifying low molecular-mass bacteriocins, we discovered that *Z.*

mobilis secretes a number of proteins and one of them exhibits an increased abundance in the supernatant after the late exponential phase (Additional file 1: Figure S1). The protein secreted was unambiguously identified by mass spectrometry sequencing of tryptic fragments (Additional file 2: Figure S2); it was Zmo0994, a functionally uncharacterized protein but with partial homology to members of group-3 of the late embryogenesis abundant (LEA) protein family (Additional file 3: Figure S3). These are associated with tolerance to dehydration in a wide range of plant species [21]. The secreted Zmo0994 protein had a signal sequence indicative of its targeting the periplasm [22].

Typically, ethanol production by *Z. mobilis* occurs as cells grow. Thus, the secretion of Zmo0994 protein during the stationary phase might suggest that it plays a role in conferring ethanol tolerance to *Z. mobilis*. We hypothesized that the increase in ethanol concentration might trigger the secretion of Zmo0994. To test the hypothesis, ethanol fermentations were performed and the extracellular protein profiles from *Z. mobilis* were analyzed. After the complete consumption of glucose at 16 h, the growth of *Z. mobilis* appeared to cease at around the 24 h time point, when the ethanol concentration reached a maximum (35.1 ± 0.3 g/L in Fig. 1a). At 24 h, the abundance of Zmo0994 protein was the highest among the secreted proteins (Fig. 1b). Subsequently, we sought to investigate whether the RNA expression level of *zmo0994* is up-regulated in the presence of ethanol stress in *Z. mobilis* using quantitative reverse transcription PCR (qRT-PCR). This qRT-PCR revealed that the expression level of *zmo0994* was significantly higher after the exposure of the *Z. mobilis* to 6% (v/v) of ethanol (Fig. 1c and Additional file 4: Figure S4). Therefore, it was confirmed that Zmo0994 expression is directly associated with ethanol stress in *Z. mobilis*.

Multi-stress tolerance increased in *E. coli* harboring *zmo0994*

Considering our previous observations, we sought to test whether Zmo0994 enhances abiotic stresses tolerance by expressing the *zmo0994* gene in *E. coli*. To demonstrate this, *E. coli* BL21(DE3) cells were transformed with either empty vector (pET21a) or recombinant plasmid (pET21a::*zmo0994*), yielding *E. coli* Emp and *E. coli* ZM, respectively. The tolerance of these transformants were tested against a wide range of abiotic stresses, including ethanol (4–8%, v/v), furfural (10–30 mM), hydroxymethylfurfural (HMF; 10–30 mM), and heat (44–48 °C). A significant difference was observed in the growth of colonies, grown at either a 10^{-1} or 10^{-2} dilution, with the *E. coli* ZM strain producing a significantly higher number of colonies compared to *E. coli* Emp (Fig. 2a). Furthermore,

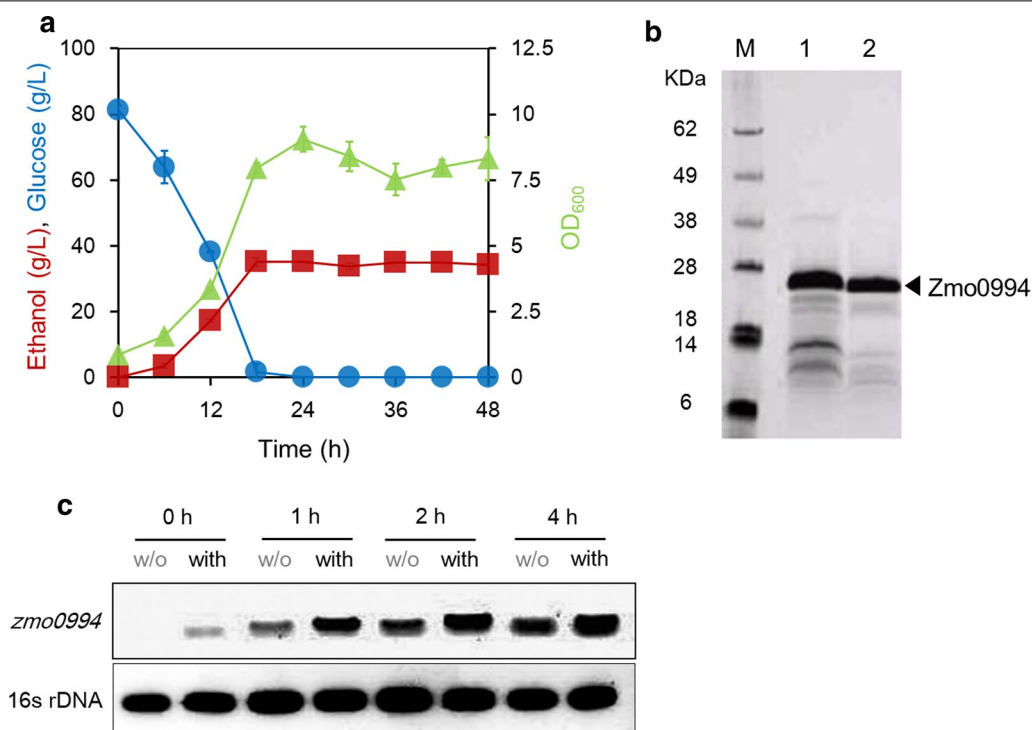


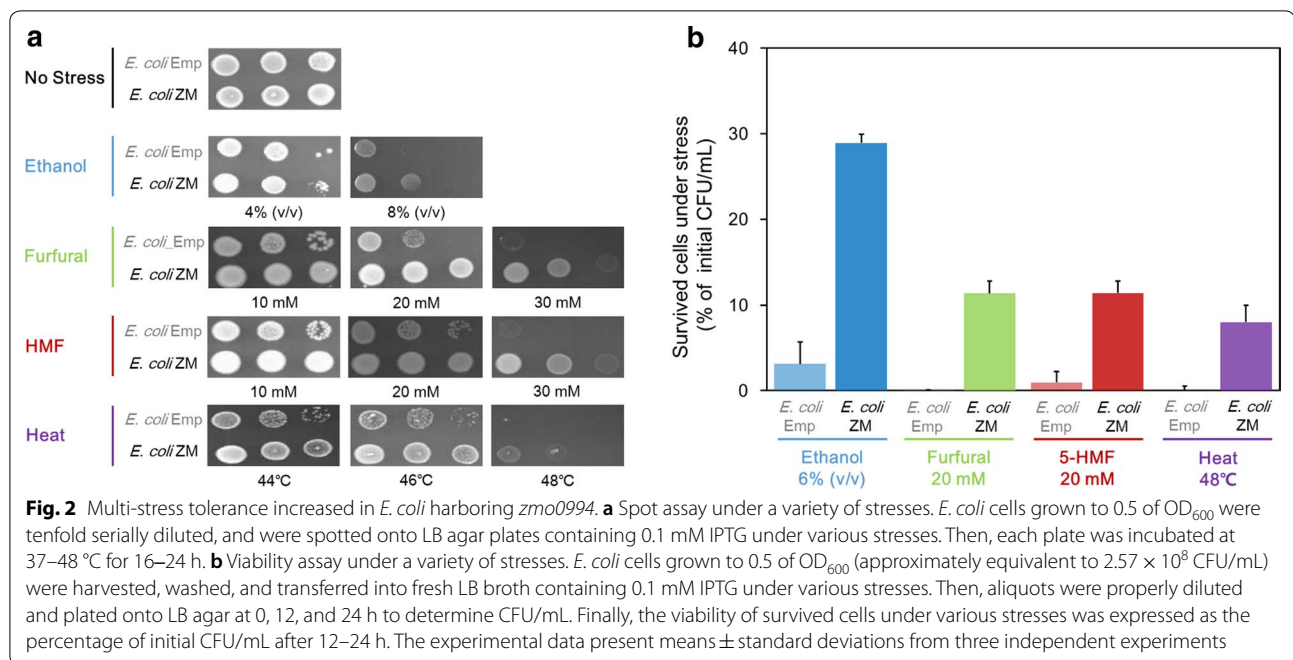
Fig. 1 Identification of Zmo0994 overproduced under stresses in *Z. mobilis*. **a** Fermentation profiles of *Z. mobilis*. Initial cell density was set to 0.5 of optical density at 600 nm (OD₆₀₀) and cell growth, glucose and ethanol concentrations were monitored during ethanol fermentation. **b** Overproduction of Zmo0994 at 24 h, when glucose was completely depleted and ethanol concentration was at the maximum. Figure was reprinted and adapted with permission from the reference [69]. **c** Quantitative RT-PCR analysis for *zmo0994* expression in the absence and presence of ethanol in *Z. mobilis*. When the cell density of *Z. mobilis* grew to 0.5 of OD₆₀₀, cells were either treated or non-treated with 6% (v/v) ethanol. Then, the total RNA was isolated from *Z. mobilis* and was converted into cDNA. Finally, PCR was performed to amplify the partial fragment of *zmo0994* (188 bp). As a housekeeping gene, partial fragment of 16 s ribosomal DNA (129 bp) was amplified as shown. The experimental data represent means \pm standard deviations from either two or three independent experiments

we sought to assess the viability of the transformant cells when exposed to different abiotic stresses: a viability assay was performed in which the survival of cells, using an equivalent initial CFU (colony-forming unit)/mL of *E. coli* ZM and *E. coli* Emp, was measured after exposure to ethanol, furfural, HMF, and heat stress for 12–24 h. Significantly, the *E. coli* ZM cells had a mean viability ratio \sim 9.3-fold higher than that of the *E. coli* Emp cells under ethanol stress (Fig. 2b). In addition, the *E. coli* ZM transformants had a significantly higher viability ratio than *E. coli* Emp transformants after exposure to 20 mM furfural, 20 mM HMF, and heat stress (Fig. 2b).

Correlation between expression level of Zmo0994 and stress tolerance in *E. coli*

Furthermore, to substantiate that Zmo0994 is responsible for multi-stress tolerance in *E. coli*, we tested whether the stress tolerance of cells is significantly affected by the expression level of Zmo0994. In this test, ethanol was used as a model chemical for multi-stress tolerance. For fine-tuning the expression level, the 5'-untranslated

region (5'-UTR) of the *zmo0994* gene was engineered [23]. Two synthetic 5'-UTRs, which were predicted to express different levels of Zmo0994, were introduced into the pET21a vector, yielding U1pET and U2pET plasmids (Fig. 3a). The resulting recombinant plasmids were transformed into *E. coli* BL21(DE3), yielding *E. coli* ZMU1 and *E. coli* ZMU2 strains, respectively. To determine the expression levels of Zmo0994 regulated by the three different 5'-UTRs, a Western-blot analysis was performed. This Western-blot analysis indicated that the Zmo0994 expression levels regulated by the three 5'-UTRs varied according to the predictive expression levels (Fig. 3b and Additional file 5: Figure S5). Subsequently, to examine the effect of variation in the expression levels of Zmo0994 on ethanol tolerance, the *E. coli* transformants were incubated in LB media containing 0–6% (v/v) ethanol (Fig. 3c). Although the *E. coli* ZM, *E. coli* ZMU1, and *E. coli* ZMU2 strains grew faster than *E. coli* Emp in the absence of ethanol, all the strains could grow up to 1.3–1.6 of OD₆₀₀ after 8 h. In the presence of 4% ethanol, all the strains expressing Zmo0994 could grow up to



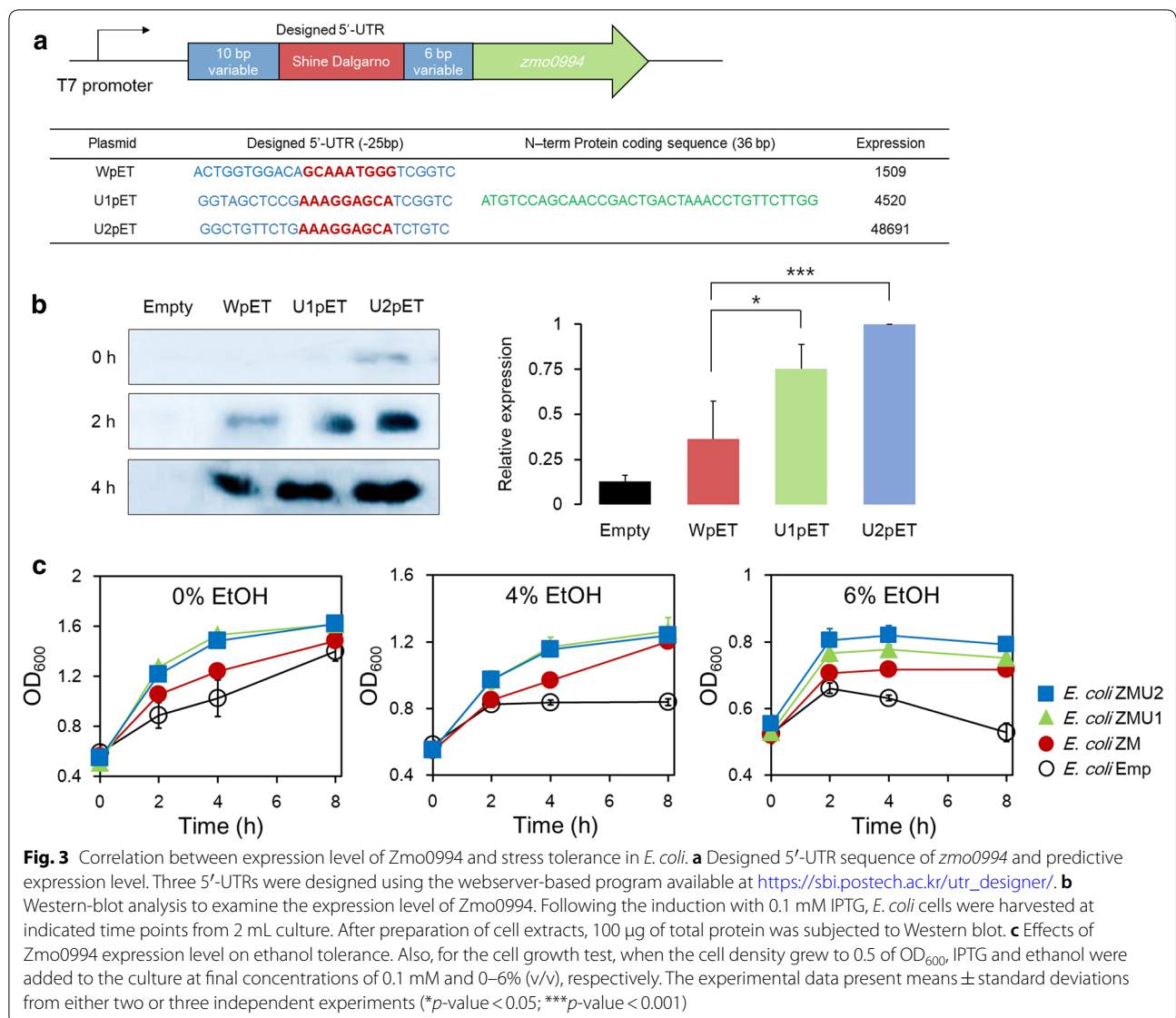
1.2 of OD₆₀₀, while the *E. coli* Emp transformants ceased to grow after 2 h, reaching 0.8 of OD₆₀₀. In 6% ethanol, the *E. coli* ZMU2 cells, which showed the highest expression level of Zmo0994, exhibited the highest growth, specifically after 2 h, while *E. coli* Emp cells exhibited an observable decrease in cell growth. Consequently, it was confirmed that an LEA-like protein, Zmo0994, from *Z. mobilis* was responsible for the multi-stress tolerance of *E. coli*.

RNA-seq-based identification of genes underlying stress tolerance in *E. coli* harboring *zmo0994*

Prior to investigating the impact of Zmo0994 on the cellular response of stress-tolerant *E. coli* ZM, we sought to identify the genes that are differentially expressed under ethanol stress (4%, v/v) in *E. coli* as a positive control, using both the *E. coli* ZM and *E. coli* Emp strains (Additional file 6: Figure S6 and Additional file 7: Tables S1 and S2). A number of well-known stress response genes were commonly up-regulated in both *E. coli* strains such as *spy* [24], *asr* [25], *yjfo*, *rrlA*, *pspG* [26], *treBC*, and *ibpAB* [9]. However, *srlBDE* genes for osmotic stress [27, 28], *yqhD* for oxidative stress response [29], *pspAD* for phage shock protein [26], as well as *nrdD* for DNA synthesis and repair were distinctly up-regulated in *E. coli* ZM. In contrast, *sodA* and *soxR* for reactive oxygen species (ROS) response [30], *aldA*, *acs*, and *glpK* for glycerol, lactate, and acetaldehyde metabolism, respectively, and *cpxP* [31], *pspB* [26], *uspAF* [32], and *gntY* [33] for stress response were up-regulated in *E. coli* Emp, but not

in *E. coli* ZM. Thus, we found that ethanol differentially induced genes for SOS response (the *sodA* and *soxR*) in *E. coli* Emp and for osmotic stress response (the *treBC* and *srlBDE*) in *E. coli* ZM (Additional file 6: Figure S6). On the other hand, the genes that were down-regulated in response to ethanol stress were mostly those coding for membrane proteins (*tsgA*, *ompGN*, and *yeaL*) and membrane transporters (*yqiG*, *garP*, *trkG*, *ycaM*, *maleK*, and *melB*) (Additional file 6: Figure S6 and Additional file 8: Tables S3 and S4).

Considering that the expression of *zmo0994* in *E. coli* caused a distinct set of genes to be expressed, we further investigated those differentially expressed genes (DEGs) in response to Zmo0994 in the absence or presence of 4% (v/v) ethanol. Specifically, we focused our analysis on >log₂ twofold genes by Zmo0994 expression, because we could not obtain a clear understanding of the functional significance in down-regulated genes (Additional file 9: Tables S5 and S6). Firstly, we collected 250 genes, which were commonly overexpressed in *E. coli* ZM compared to *E. coli* Emp in the presence and absence of ethanol stress (Additional file 10: Tables S7 and S8). Among these, we found that Zmo0994 triggered genes involved in a variety of stresses and genes inducible under oxygen-deprivation conditions: for instance, *polA* [34] and *nfsA* [35] for oxidative stress; *psd*, *prfAB*, and *plsB* regulated by RpoE (σ₂₄) for heat stress [36, 37]; *hflKX*, *htpG*, and *dnaJ* regulated by RpoH (σ₃₂) for protease or chaperon [36, 37]; *cfa* [38], *yjiT* (UV), *dps* (nutrient) [39], *mutS* (DNA repair) [40], *uvrB* (UV) [41], and *cspD* [42] for general stresses;



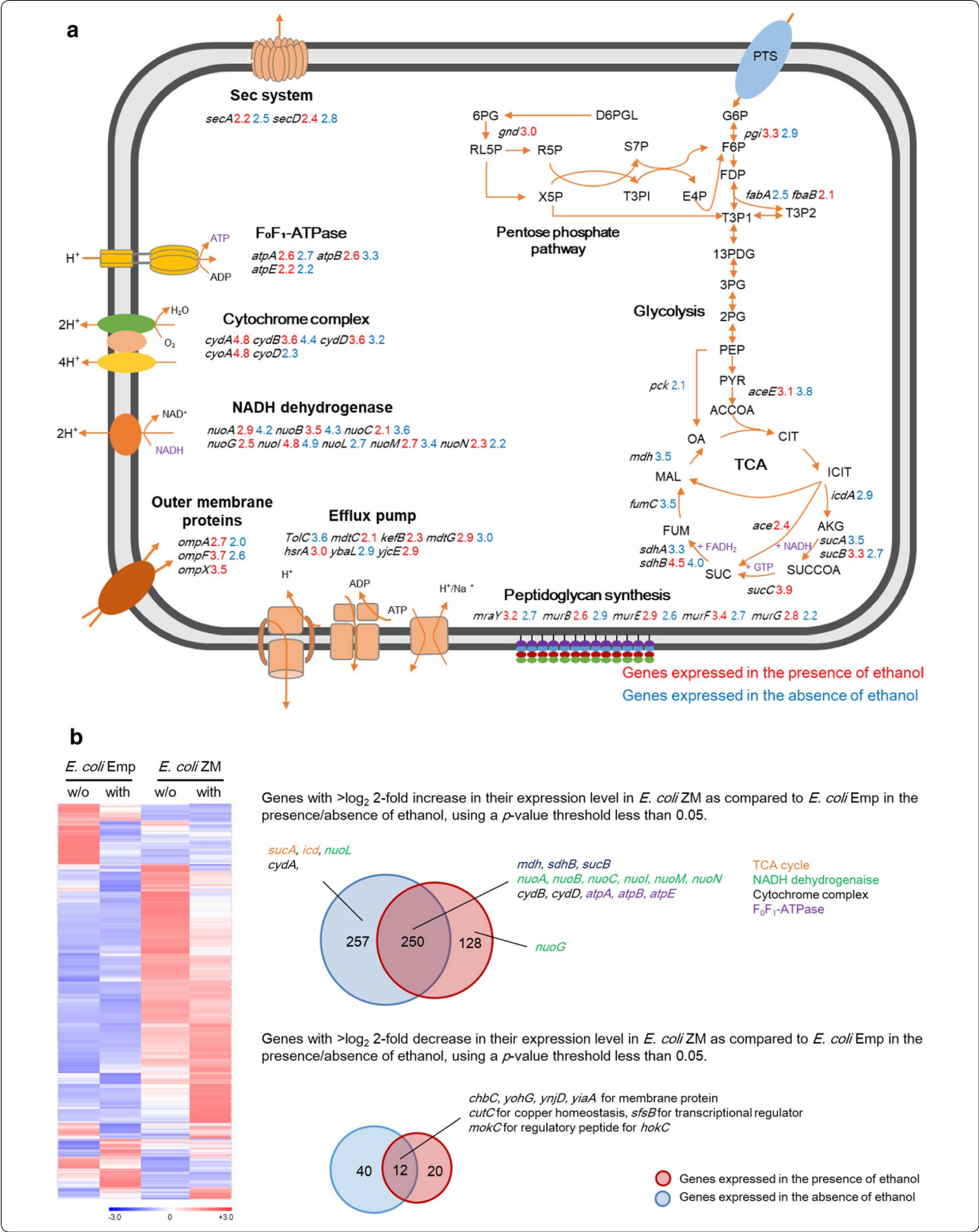
glpT, *dcuA*, *aceE*, *fadAH*, and *pflA* for oxygen deprivation responses.

We further found that Zmo0994 triggered the expression of gene clusters involved in aerobic respiration (Fig. 4a): five genes for the TCA cycle (*sdhB*, *mdh*, *sucAB*, and *icd*); eight genes for NADH dehydrogenase (*nuoABCGILMN*); three genes for cytochrome ubiquinol oxidase

(*cydABD*); and three genes for F₀F₁-ATPase (*atpABE*) (Fig. 4). However, fumarate reductase complex, which is one of the electron transport chains in *E. coli*, was not up-regulated. Among the 19 genes, encoding for the components of ATP synthesis in aerobic respiration, the expression of 6 genes (*nouI*, *sdhB*, *cydB*, *cydD*, *nuoB*, and *sucB*) was noticeably observed to be increased as much as

(See figure on next page.)

Fig. 4 Transcriptome analysis to understand cellular mechanism of ethanol tolerance by Zmo0994. **a** A graphical representation of DEGs in *E. coli* ZM as compared to *E. coli* Emp in the presence/absence of ethanol. **b** A putative regulatory pathway of *E. coli* in response to Zmo0994. The pathway was constructed based on the functional clustering of DEGs (Additional file 16: Figure S11). *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *FDP* fructose 1,6-biphosphate, *T3P1* D-glyceraldehyde 3-phosphate, *T3P2* glyceraldehyde 3-phosphate, *13PDG* 1,3-biphospho-D-glycerate, *PYR* pyruvate, *ACCOA* acetyl coenzyme A, *ICIT* isocitrate, *AKG* alpha-ketoglutarate, *SUCCOA* succinyl coenzyme A, *SUC* succinate, *FUM* fumarate, *MAL* malate, *OA* oxaloacetate, *6PG* 6-phosphogluconate, *RL5p* ribulose 5-phosphate



\log_2 threefold or greater: 4.84, 4.51, 3.59, 3.58, 3.45, and 3.31, respectively. The *sucA*, *icd*, *nuoL*, and *cydA* genes were up-regulated only in the absence of ethanol stress, while the *nuoG* gene was up-regulated only in presence of ethanol stress by Zmo0994. Additionally, genes for efflux pumps (including the *tolC*) and for cell wall biogenesis (including the *mur* operon) were up-regulated. Finally, among the up-regulated 635 genes by Zmo0994 in the presence or absence of ethanol, those expected to be responsible for stress tolerance are summarized in Fig. 4b.

ATP/ADP ratio

Based on the transcriptome analysis (Fig. 4a), indicating that gene clusters involved in ATP synthesis were highly up-regulated in response to Zmo0994, it was hypothesized that Zmo0994 might improve ATP production, resulting in enhancement of cell viability under a variety of stresses. Thus, we measured intracellular ATP and ADP concentrations in the *E. coli* ZM and *E. coli* Emp cells in the absence and presence of ethanol. In results, time course measurements of the intracellular ATP/ADP ratio showed that *E. coli* ZM strain exhibits higher ATP/ADP ratio than *E. coli* Emp strain at 4 h regardless of ethanol stress (Fig. 5). More specifically, ATP/ADP ratio of *E. coli* ZM was observed to be 4.3- and 7.7-fold higher than that of *E. coli* Emp in

the absence and presence of ethanol stress, respectively. Thus, these results demonstrated that the overexpression of Zmo0994 increased the intracellular ATP/ADP ratio, resulting in protection of cells against ethanol stress.

In vivo validation of identified genes for stress tolerance

Among the genes identified from the transcriptome analysis, we focused on the key genes clusters, which were distinctly up-regulated by Zmo0994. Thus, the genes for ATP synthesis under aerobic respiration, multi-drug efflux pump, and cell wall biogenesis were targeted to elucidate the mechanism of stress tolerance conferred by Zmo0994. More specifically, they were seven genes for the TCA cycle, eight genes for NADH dehydrogenase, four genes for cytochrome complex, two genes for FoF1-ATPase, six genes for multi-drug efflux pump, and six genes for cell wall biogenesis (Additional file 11: Table S9). For the stress tolerance tests, each of the 33 genes were independently expressed in *E. coli* BL21(DE3), with the transformants grown in the presence or absence of 4% (v/v) ethanol and HMF (10 mM). The *E. coli* Emp and *E. coli* ZM strains were used as the negative and positive controls for the tolerance test. Firstly, we verified that *E. coli* ZM exhibited >10- and >2-fold increase in the fitness compared to that of *E. coli* Emp under ethanol and HMF stress, respectively. Subsequently, we found that 20 and 14 out of the 33 tested genes significantly enhance ethanol and HMF tolerance, respectively, when expressed in *E. coli* BL21(DE3) (Fig. 6 and Additional file 12: Figures S7 and S8). Among them, the fitness of six genes (*sucB*, *icdA*, *cydA*, *cyoB*, *kefB*, and *murC*) showed >10-fold increase under ethanol stress compared to that of *E. coli* Emp, while the fitness of nine genes showed >2-fold increase under HMF compared to that of *E. coli* Emp (Fig. 6). Notably, overexpression of 11 genes in *E. coli* significantly increased both ethanol and HMF tolerance. These are *sucB* (dihydrolipoyltranssuccinylase), *icdA* (isocitrate dehydrogenase), *nuoE* (NADH:ubiquinone oxidoreductase, chain E), *nuoG* (NADH:ubiquinone oxidoreductase, chain E), *cydD* (glutathione/L-cysteine exporter), *atpA* (ATP synthase F1 complex, α subunit), *tolC* (outer membrane channel), *kefB* (K^+ : H^+ antiporter), *murC* (UDP-N-acetylmuramate-alanine ligase), *murD* (UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase), and *murF* (D-alanyl-D-alanine-adding ligase).

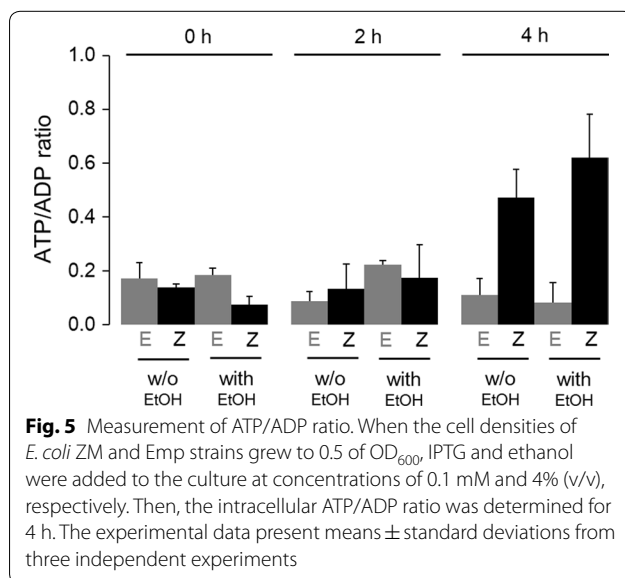
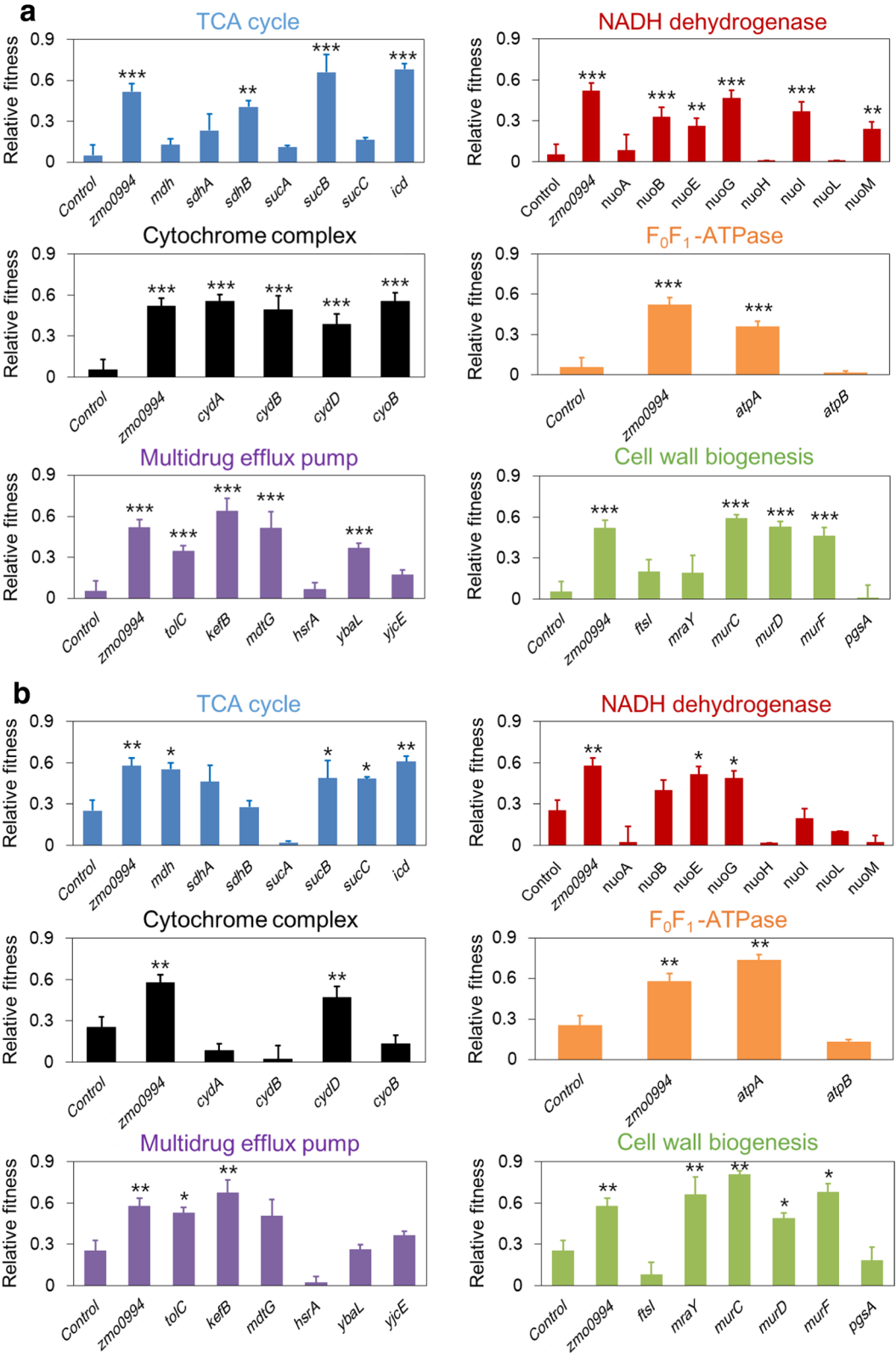


Fig. 5 Measurement of ATP/ADP ratio. When the cell densities of *E. coli* ZM and Emp strains grew to 0.5 of OD_{600} , IPTG and ethanol were added to the culture at concentrations of 0.1 mM and 4% (v/v), respectively. Then, the intracellular ATP/ADP ratio was determined for 4 h. The experimental data present means \pm standard deviations from three independent experiments

(See figure on next page.)

Fig. 6 In vivo validation of identified genes for stress tolerance. The relative fitness of *E. coli* harboring each gene involved in oxidative phosphorylation, electron transfer chain, efflux pump, and cell wall biogenesis under **a** ethanol (4%, v/v) and **b** HMF (20 mM) stresses. *E. coli* BL21(DE3) harboring each gene was incubated in 100 mL of LB broth at 37 °C for ethanol tolerance and was incubated in spectrophotometer (200 μ L scale) for HMF tolerance. Finally, the relative fitness was calculated as described in “Methods”. The experimental data present means \pm standard deviations from three independent experiments (** p -value < 0.01; *** p -value < 0.001)



Discussion

Herein, we have discovered that Zmo0994 from the bacterium *Z. mobilis*, a novel LEA-like protein that is utilized by plant cells in protecting them against various stresses, such as dehydration, can confer tolerance to various abiotic stresses when expressed in *E. coli*. This is an important discovery, since it could provide a strategy for engineering an increase in the tolerance to abiotic stresses of bacterial strains used for fermentation processes. For example, a number of toxic compounds are produced during the pretreatment of lignocellulose, which act as inhibitors that hinder the fermentation process. Interestingly, while our initial expectation was that Zmo0994 would act to physically protect the cells from abiotic stresses similar to LEA proteins in plants, we discovered that it could regulate the expression of genes (e.g., genes for ATP synthesis) involved in conferring tolerance to abiotic stresses.

LEA proteins from plants have been shown to play a cellular protective role against hypersalinity, freezing, and temperature stresses [18, 19]. It is proposed that under abiotic stress conditions, LEA may act as a chaperone to suppress desiccation-induced protein aggregation, a role that is likely potentiated by the interaction with non-reducing sugars such as trehalose and sucrose [21]. These sugars can also protect lipid membranes from abiotic stress that causes solute leakage and membrane protein aggregation [43]. The protective effect is due to the sugars forming a glassy matrix that prevents mechanical disruption and denaturation of membrane proteins. It has been shown that sucrose glasses are stabilized in vitro by interaction with LEA proteins [46]. A recent phylogenetic analysis suggests that the LEA protein from plants may have ancestral origins in the domains *Bacteria* and *Archaea* with acquisition endosymbionts or horizontal gene transfer [44]. Thus, it is not surprising that overexpression of LEA protein from plants increased tolerance against abiotic stresses in microorganisms [20, 45].

LEA proteins have been grouped together with other osmotic stress-induced proteins from *Saccharomyces cerevisiae* and *E. coli* into a class of proteins termed hydrophilin, based on their high hydrophilicity index (>1.0) and glycine content (>6%) [46, 47]. Among these proteins, ribosome modulation factor (RMF), accumulates in *E. coli* upon growth transition from the exponential to the stationary phase [48], suggesting a role in stress tolerance like that of Zmo0994. In yeast, the small heat shock protein 12 (HSP12) was first identified as a novel hydrophilin-like protein [49] and it has been generally regarded as a membrane-associated chaperone that confers tolerance against oxidative, thermal, and osmotic stresses [50, 51]. In this study, it was found that Zmo0994 can be grouped into the hydrophilin class of proteins

[46], when its N-terminal signal peptide, consisting of the first 33 amino acids of its sequence, was removed (Fig. 7a). Interestingly, a recent evolutionary data on LEA-like proteins from bacteria indicate that only 12 out of 26 LEA proteins have a signal peptide responsible for protein secretion [52]. To test whether the signal peptide of Zmo0994 affects ethanol tolerance, *E. coli* ZMsig(-) harboring N-term-truncated *zmo0994* was constructed and was found to exhibit ethanol tolerance (Fig. 7b). Thus, establishing that Zmo0994 plays a role in the cytoplasm rather than, or in addition, to any role in the cell membrane, subsequently, we confirmed that GFP-fused Zmo0994 expressed in *E. coli* was localized in the cytoplasm rather than the cell membrane (Additional file 13: Figure S9). Because Zmo0994 induces up-regulation of a number of genes, it was hypothesized that it might function as an RNA chaperone. However, we cannot find any evidence supporting this notion in the experiment substituting cold shock protein [53] with Zmo0994 (Additional file 14: Figure S10).

Previous studies showed that ethanol mainly attacks the cell membrane and increases oxidative stress,

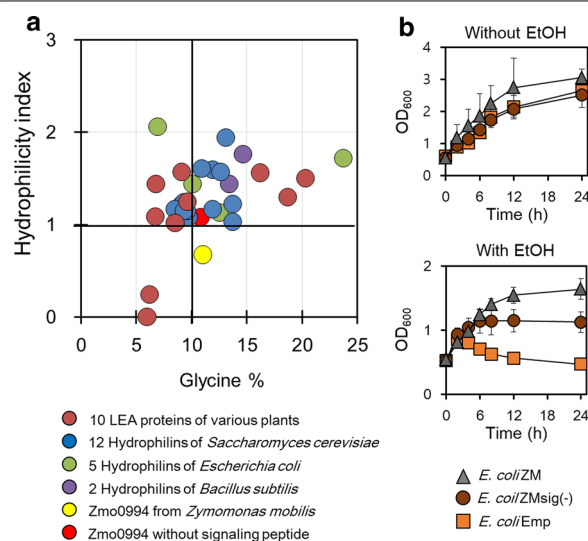


Fig. 7 *E. coli* harboring N-terminal truncated *zmo0994* exhibited ethanol tolerance. **a** Plot construction based on hydrophilicity index and percentage of glycine of Zmo0994, hydrophilins from microbes, and LEA proteins from plants. The annotated hydrophilins were acquired from Garay-Arroyo et al. [46] and of LEA proteins from plants were used as a control set (Additional file 17: Note S1), and the hydrophilicity index was determined using webserver-based program available at <https://web.expasy.org/protscale> [70]. **b** *E. coli* harboring signal peptide truncated *zmo0994* [*E. coli* ZMsig(-)] still exhibits ethanol tolerance. For the cell growth test, when the cell density grew to 0.5 of OD₆₀₀, IPTG and ethanol were added to the culture at final concentrations of 0.1 mM and 4% (v/v), respectively. The experimental data present means ± standard deviations from three independent experiments

resulting in reduced ATP production by aerobic respiration that eventually leads to reduced biosynthesis of macromolecules and proliferation [54–56]. Notably, our RNA-seq results indicated that those genes up-regulated in response to Zmo0994 are mainly involved in ATP synthesis by aerobic respiration (Fig. 4). Indeed, 13 out of 21 genes involved in ATP synthesis significantly enhanced ethanol tolerance when expressed in *E. coli* (Fig. 6a). Thus, these genes, up-regulated in response to Zmo0994, might compensate for the decreased aerobic ATP synthesis due to increased membrane fluidity and ROS level under ethanol stress. Consistent with this interpretation, the intracellular ATP/ADP ratio of *E. coli* ZM was significantly higher than that of *E. coli* Emp (Fig. 5), indicating that ATP synthesis might be a key cellular response for stress tolerance. Although most of the tested genes for ATP synthesis in this study were previously reported to be responsible for ethanol tolerance based on the transcriptome and proteome analysis [54, 57, 58], this is the first study to verify their role in ethanol and HMF tolerance. Considering that ATP participates in many cellular processes as a major substrate for energy production, manipulation of ATP supply could be a powerful tool to enhance tolerance to abiotic stresses [59]. Herein, we also established that three genes for multi-drug efflux pumps (*tolC*, *kefB*, and *mdtG*) and four genes for cell wall biogenesis (*mraY* and *murCDF*) enhanced ethanol tolerance. Specifically, *murCDF* genes for peptidoglycan synthesis are involved in ATP binding and hydrolysis. Several efflux pumps, such as AcrAB-TolC in *E. coli*, were experimentally verified to enhance solvent tolerance by exporting a broad range of chemicals [8]. The *murD* gene product for peptidoglycan biosynthesis was confirmed to be beneficial in conferring ethanol stress [60], while the *murCF* and *mraY* genes were firstly verified to be responsible for stress in this study.

On the other hand, a total of 70 genes commonly up-regulated by ethanol stress in both *E. coli* ZM and *E. coli* Emp were mainly involved in protein folding (chaperone), ROS detoxification, DNA damage repair, anaerobic metabolism, and rRNA processing (16S and 23S) (Additional file 4: Figure S4). Among these, several genes could be genetic targets for abiotic stress tolerance, such as phage shock protein (Psp) and DNA-binding protein from starved cells (Dps). The *psp* operon is known to play a key role in maintaining proton motive force by suppressing membrane leakage under nutrient- or energy-limited conditions [26]. Thus, Psp is regarded as a different type of envelope stress responses, which is distinct from periplasmic response proteins (e.g., CpxP) induced by ethanol [31, 61]. Especially, *pspA*, the regulator of *psp* operon, is a notable gene in that it could be a potential target for multi-stress tolerance in the

development of industrial microorganism, because toxic compounds typically attack cell membranes, resulting in an increase of membrane fluidity [61]. Meanwhile, Dps binds the genomic DNA non-specifically during stationary phase, forming a highly ordered and stable Dps–genomic DNA complex, which is condensed and protected from diverse damages [62]. It protects DNA from oxidative damage by sequestering intracellular Fe^{2+} ion and storing it in the form of Fe^{3+} oxyhydroxide mineral, which can be released after reduction. One hydrogen peroxide oxidizes two Fe^{2+} ions, preventing hydroxyl radical production by the Fenton reaction [63]. In addition, Dps protects the cell from UV and gamma irradiation, iron and copper toxicity, thermal stress, and acid shocks [62, 64]. Another interesting observation in this study is that approximately 11% of the genes up-regulated are rRNA components (*rrsCDHG*, *rrdACDH*) by ethanol stress. Recently, it was reported that an rRNA variant (e.g., *rrsH*) affects the expression of general stress response protein regulated by RpoS (σ^{38}) [65]. Thus, manipulation of genes coding the components for transcription and translation could be a potential tool for stress tolerance [66, 67].

Conclusions

The development of robust microbes for lignocellulose-based processes is essential for cost reduction in industrial-scale fermentation. Thus, a variety of efforts from metabolic engineering to omics analyses have been employed to date. In this study, we identified the functionally uncharacterized protein, Zmo0994, a novel small LEA-like protein from *Z. mobilis*, as a putative regulator of gene clusters, involved in aerobic respiration for ATP synthesis. Its major effect was a significant enhancement of tolerance to abiotic stresses, such as ethanol and inhibitors derived from lignocellulose pretreatment, when expressed in *E. coli*. In this regard, this discovery has significant implications in the development of robust microbes for efficient industrial fermentation processes.

Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table S10 (Additional file 15). *Z. mobilis* subsp. *mobilis* ZM4 (ATCC 31,821) was grown at 30 °C and 80–200 rpm in RM medium (20 g/L of glucose, 10 g/L of yeast extract, 2 g/L of KH_2PO_4 ; pH 6.0). *E. coli* BL21(DE3) was used as the host for the expression of a target protein cloned in pET21a vector. *E. coli* strains were incubated at 37 °C in Luria–Bertani (LB) supplemented with ampicillin at 100 µg/mL.

Fermentation and purification of extracellular proteins from *Z. mobilis*

Z. mobilis cells grown to the exponential growth phase (2.0 of OD₆₀₀) in RM broth were harvested and transferred into 1 L of RM containing glucose 80 g/L. The initial cell density was adjusted to approximately 0.8 of OD₆₀₀. During the incubation at 30 °C and 80 rpm without a gas fed, cell growth was monitored by OD₆₀₀ and glucose and ethanol concentrations were measured using a high-performance liquid chromatography (HPLC; Waters, Milford, MA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The column temperature was set at 65 °C and 0.5 mM H₂SO₄ solution was used as the mobile phase at a constant flow rate of 0.5 mL/min. Peaks were detected by a refractive index detector (RID) at 55 °C and were quantified according to calibration curves of glucose and ethanol.

For the purification of extracellular proteins, the supernatant from 1 L of *Z. mobilis* culture was separated every 2–4 h by centrifuging at 8000 rpm for 20 min at 4 °C and was passed through a 0.22 µm sterile vacuum filter. The supernatant was mixed with either 1 mL of SP-sepharose or Q-sepharose fast flow resin (GE healthcare, Marlborough, MA) for anion or cation exchange chromatography, respectively, at 4 °C for 1 h. Then, the mixture was poured into a glass column and immobilized proteins were eluted with a 0–1.0 mM NaCl gradient in 50 mM phosphate buffer (pH 7.0). Finally, the purified proteins were visualized by SDS-PAGE analysis.

Mass spectrometry analysis and protein identification

Following an SDS-PAGE analysis, the bands corresponding to proteins of interest including Zmo0994 were cut out of the gel, equilibrated in 50 µL of 50 mM ammonium bicarbonate, and subsequently alkylated with 10 mM dithiothreitol (DTT) and 100 mM iodoacetamide (IAA), followed by destaining and desiccation with acetonitrile. Gel plugs were rehydrated with 50 mM ammonium bicarbonate containing 6.6% (w/v) trypsin (Promega, Madison, WI) and digested overnight. Peptides were extracted using 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) into a final volume of 50 µL, and the resulting extracts were freeze-dried and resuspended in 10 µL of 0.1% formic acid. After samples were washed in situ with 0.1% TFA and left to dry again, 0.2 µL of matrix (e.g., α-cyano-4-hydroxycinnamic acid in nitrocellulose/acetone) was applied to a mass spectrometer target plate. Then, MALDI-TOF PMF was performed using a Voyager-DE™ STR BioSpectrometry™ Workstation (Applied Biosystems, Warrington, UK). De-isotoped and calibrated spectra were used to generate peak lists, which were searched using MASCOT (www.matrixscience.com)

mass spectrometry database search software to identify the proteins.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

When *Z. mobilis* cells were grown to the early exponential phase at 0.5 of OD₆₀₀, cells were either treated or non-treated with ethanol (6%, v/v) to observe the expression patterns of *zmo0994*. Total RNA was isolated from 10 mL culture using RNeasy Mini Kit (Qiagen, Hilden, Germany) and the isolated RNA was quantified using NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific, Wilmington, DE). Then, reverse transcription reactions were applied for cDNA synthesis with 1 µg of total RNA with random hexamer (SuperScript™ II Reverse Transcriptase; Thermo Fisher Scientific). Finally, *zmo0994* and 16S rRNA genes were amplified from synthetic cDNA as a template with the designed primers: ZM_RT_Fwd and ZM_RT_Rev for *zmo0994*; ZM_16s_Fwd and ZM_16s_Rev for 16 s rDNA of *Z. mobilis* (Additional file 15: Table S10).

Assays for tolerance and viability

For spot assay, overnight culture of *E. coli* ZM and *E. coli* Emp strains were diluted at 0.02 of OD₆₀₀ into 100 mL of LB broth and were incubated at 37 °C. When *E. coli* cells were grown to the early exponential phase at 0.5 of OD₆₀₀, 5 µL of tenfold serially diluted cell suspensions was spotted onto LB-ampicillin plate containing 4–8% (v/v) of ethanol, 10–30 mM of furfural, and 10–30 mM of HMF with addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Then, the plates were incubated at 37 °C and examined for 16–36 h. Tolerance of *E. coli* ZM to thermal stress was examined in a similar manner on LB plates at 44–48 °C. In addition, for viability assay, *E. coli* cells were grown to 0.5 of OD₆₀₀, corresponding to approximately 2.57×10^8 CFU/mL, and they were exposed to 6% (v/v) ethanol, 20 mM furfural, 20 mM HMF, and 48 °C for 12–48 h. Viable cells were determined by colony counting for 24 h at 12 h interval. Finally, the viability of survived cells under various stresses was expressed as the percentage of initial CFU/mL after 12–24 h.

Whole-transcriptome shotgun sequencing (RNA-seq) and analysis

When *E. coli* ZM and *E. coli* Emp cells were grown to the early exponential phase at 0.5 of OD₆₀₀, cell cultures were either treated or non-treated with ethanol (4%, v/v). After 4 h, cell pellets from the cultures were collected by centrifuging at 8000g for 10 min at 4 °C and washed twice with ice-cold 50 mM phosphate-buffered saline (PBS). Then, the pellets were quickly placed in liquid nitrogen and

were sent to DNALink (Seoul, Korea) for RNA-seq. RNA extraction, rRNA removal, RNA fragmentation, cDNA synthesis, adapter ligation, and PCR amplification were performed to construct a cDNA library. Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) verified the quality of the amplified library. Sequencing was performed using Illumina HiSeq 2500 system (Illumina, San Diego, CA) with 200 bp read length. Clean reads for each sample were obtained, filtered, and mapped to the reference genome of *E. coli* K12 MG1655 by Tophat (version 2.0.13) [68]. The aligned results were added to Cuffdiff (version 2.2.0; <https://cole-trapnell-lab.github.io/cufflinks/cuffdiff/>) for library normalization and for identification of differentially expressed genes (DEGs). The significance of differences in gene expression was defined as $p < 0.05$ and $> \log_2$ twofold. The gene ontology analysis was performed with DAVID (version 6.8; <https://david.abcc.ncifcrf.gov/>).

Modification of 5'-untranslated region (5'-UTR)

The 5'-UTR variants of *zmo0994* were constructed as described previously²³. Briefly, UTR Designer (https://sbi.postech.ac.kr/utr_designer) was used to predict the expression level of *zmo0994* with a particular mRNA sequence in 5'-UTR. The entire pET21(a)::*zmo0994* plasmid was used as the PCR template with 5'-phosphorylated primers (Z_U1_Forward, Z_U2_Forward, and UTR_Reverse) to generate the 5'-UTR variants (Additional file 15: Table S10). The resulting PCR products were purified via gel extraction using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and the template DNA was eliminated by treatment with DpnI (NEB) at 37 °C for 3 h. The PCR products were blunt-end ligated with T4 DNA ligase (NEB) at 16 °C for 4 h and used to transform *E. coli* DH5 α strain. Purified plasmids from transformants were sequenced by Macrogen (Seoul, Korea) and the screened plasmids containing the *zmo0994* gene with the targeted 5'-UTR sequence were transformed into *E. coli* BL21(DE3).

Western blot

After *E. coli* strains grown at 0.5 of OD₆₀₀ were treated with 0.1 mM of IPTG in 10 mL, cell pellets from 2 mL of cell cultures were collected at various time points and resuspended in 100 μ L of lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 tablet of protease inhibitor (Roche, Mannheim, Germany)]. The resuspended cells were disrupted using glass beads (425–600 μ m size; Sigma-Aldrich, St. Louis, MO) and debris was removed by centrifuging at 14,000 rpm for 15 min at 4 °C. The supernatant proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF)

membrane (Bio-Rad, Hercules, CA). For detecting His-tagged Zmo0994, anti-6 \times histidine antibody raised in rabbit (Santa Cruz Biotechnology, Dallas, TX) against the Zmo0994 (in 1:10,000 dilution) and a goat anti-rabbit antibody (in 1:20,000 dilution) were used. Finally, protein bands were visualized by detection of horseradish peroxidase-conjugated secondary anti-rabbit antibodies. The band intensities were quantified with NIH image J (Version 1.61; National Institutes of Health, Bethesda, MD).

Measurement of relative fitness

Overnight culture of *E. coli* harboring each gene was diluted at 0.02 of OD₆₀₀ into 100 mL of LB broth and incubated at 37 °C. When the cell density reached 0.5 of OD₆₀₀, IPTG and ethanol were added to the culture at final concentrations of 0.1 mM and 4% (v/v), respectively. Then, cell growth was monitored by measuring OD₆₀₀ for 24 h at 2 h intervals. Finally, relative fitness was calculated by the following equation:

Relative fitness

$$= \frac{\ln\left(\frac{\text{OD}_{600} \text{ at } 16 \text{ h}}{\text{OD}_{600} \text{ at } 0 \text{ h}}\right) \text{ in the presence of ethanol (4\%, v/v)}}{\ln\left(\frac{\text{OD}_{600} \text{ at } 16 \text{ h}}{\text{OD}_{600} \text{ at } 0 \text{ h}}\right) \text{ in the absence of ethanol}}$$

Finally, Student's *t*-test was performed using STATISTICA 7 for the statistical analysis.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13068-020-01790-0>.

Additional file 1: Figure S1. SDS-PAGE analysis of extracellular proteins produced by *Zymomonas mobilis*.

Additional file 2: Figure S2. MASCOT search results from the MS-MS data generated for Zmo0994 (uncharacterized protein)

Additional file 3: Figure S3. Blast results of Zmo0994 (UniProt accession number: Q5NNU2). a. Q9LF88, LEA protein from *Arabidopsis thaliana*; b. A0A2K3MNT4, Group 3 LEA protein from *Trifolium pretense*.

Additional file 4: Figure S4. Quantitative RT-PCR analysis of *zmo0994* expression in *Z. mobilis* in both the absence and presence of ethanol.

Additional file 5: Figure S5. Western-blot results showing the expression level of Zmo0994.

Additional file 6: Figure S6. Differentially expressed genes of *E. coli* ZM and *E. coli* Emp in the presence of 4% (v/v) compared to those in the absence of ethanol.

Additional file 7: Table S1. Genes with $> \log_2$ twofold increase in their expression level in *E. coli* ZM in the presence of 4% (v/v) ethanol compared to in the absence of ethanol, using a *p*-value threshold less than 0.05;

Table S2. Genes with $> \log_2$ twofold increase in their expression level in *E. coli* Emp in the presence of 4% (v/v) ethanol compared to in the absence of ethanol, using a *p*-value threshold less than 0.05.

Additional file 8: Table S3. Genes with $> \log_2$ twofold decrease in their expression level in *E. coli* ZM in the presence of 4% (v/v) ethanol compared to in the absence of ethanol, using a *p*-value threshold less than 0.05;

Table S4. Genes with $> \log_2$ twofold decrease in their expression level in *E.*

coli Emp in the presence of 4% (v/v) ethanol compared to in the absence of ethanol, using a *p*-value threshold less than 0.05.

Additional file 9: Table S5. Genes with $> \log_2$ twofold decrease in their expression level in *E. coli* ZM as compared to *E. coli* Emp in the presence of ethanol (4%, v/v), using a *p*-value threshold less than 0.05; **Table S6.** Genes with $> \log_2$ twofold decrease in their expression level in *E. coli* ZM as compared to *E. coli* Emp in the absence of ethanol, using a *p*-value threshold less than 0.05.

Additional file 10: Table S7. Genes with $> \log_2$ twofold increase in their expression level in *E. coli* ZM as compared to *E. coli* Emp in the presence of ethanol (4%, v/v), using a *p*-value threshold less than 0.05; **Table S8.** Genes with $> \log_2$ twofold increase in their expression level in *E. coli* ZM as compared to *E. coli* Emp in the absence of ethanol, using a *p*-value threshold less than 0.05.

Additional file 11: Table S9. Tested 33 genes, up-regulated in the absence or presence of 4% (v/v) ethanol with $> \log_2$ twofold change in their expression level by Zmo0994 expression.

Additional file 12: Figure S7. Growth profiles of *E. coli* strains harboring the indicated gene in the absence and presence of ethanol (4%, v/v); **Figure S8.** Growth profiles of *E. coli* strains harboring the indicated gene in the absence and presence of 10 mM HMF.

Additional file 13: Figure S9. Confocal microscopy analysis for the localization of GFP-fused Zmo099.

Additional file 14: Figure S10. Results of substitution of cold shock protein with Zmo0994 for RNA chaperone test.

Additional file 15: Table S10 Bacterial strains, plasmids, and primers used in this study

Additional file 16: Figure S11. Heat map of functionally classified DEGs by Zmo0994

Additional file 17: Note S1. Amino acid sequences of hydrophilins from microorganisms and LEA proteins from plants.

Abbreviations

LEA: Late embryogenesis abundant; ATP: Adenosine triphosphate; HMF: 5-Hydroxymethylfurfural; gTME: Global transcription machinery engineering; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; CFU: Colony-forming unit; 5'-UTR: 5'-Untranslated region; ADP: Adenosine diphosphate; DEG: Differentially expressed genes; NADH: Nicotinamide adenine dinucleotide hydrogen; RMF: Ribosome modulation factor; HSP12: Heat shock protein 12; RNA: Ribonucleic acid; Psp: Phage shock protein; Dps: DNA-binding protein; UV: Ultraviolet; RM: Rich medium; HPLC: High-performance liquid chromatography; RID: Refractive index detector; IPTG: Isopropyl β -D-1-thiogalactopyranoside; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Authors' contributions

KHK designed the research, supervised the project, and wrote the manuscript. JY designed the research and contributed to the writing of the manuscript. JY, HEK, YHJ, and DHK performed experiments and analyzed the data. JK generated and provided analytical tools. ARW aided in interpreting the results and provided critical revision. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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